Formation of MethylGlyoxal Induced, Partially De-Aggregated Soluble Advanced Glycation Endproducts of Human Fibrinogen

Short Title: Glycation of Human Fibrinogen by MethyGlyoxal

Lasker S. Lasker; George W. Dombi; and Joel A. Dain*

Department of Chemistry, University of Rhode Island, Kingston, RI 02881, USA

*jdain@chm.uri.edu

Abstract

Advanced glycation endproducts (AGEs), a heterogeneous group of molecules, are formed from the non-enzymatic reaction of reducing sugars or their metabolites with free amino acids in plasma or amino groups of proteins, lipids or nucleic acids. AGEs can accumulate in plasma and tissues. Once formed, they can contribute to the pathology of a broad range of conditions including diabetes, Alzheimer's disease, cataract formation and coronary heart disease. The objective of this project was to demonstrate the in-vitro formation of advanced glycation endproducts by the reaction of human fibrinogen with MethylGlyoxal (MG), a reactive sugar metabolite. Human fibrinogen (hFib) at physiological concentrations was glycated with MG at high, observable concentrations. This showed a time dependent, hyperchromic shift in the UV absorbance at 280 nm, which indicated an increase in electron stacking suggesting an increase in adduct formation. Furthermore, the λ_{max} underwent a hypsochromic shift changing the wavelength from ~280 nm to ~265 nm after 21 days of incubation, further suggesting an increase in adduct formation. Results from HPLC and UV analysis supported the formation of a Partially De-Aggregated Soluble Advanced glycation product, (PDASA). This re-solublized aggregate may be the result of a reversible clot formation model which would be useful in subsequent experiments on the thermodynamics of human fibrinogen conformation changes.

Keywords

Fibrinogen; Methylglyoxal; Glycation; HPLC; Hyperchromic Shift; Hypsochromic Shift; Circular Dichroism (CD); Aggregated AGE Product; in Vitro Maillard Reaction

Introduction

Amino groups of protein side chains can nonenzymatically react with the carbonyl groups of sugars and some sugar metabolites to form a Schiff base that can further interact and rearrange into various Amadori products. These Schiff base and Amadori products can undergo various rearrangements, including oxidation, dimerization or polymerization (Figure 1) to form a heterogeneous class of fluorescence compounds known as Advanced Glycation End Products (AGEs) (Maillard, 1912; Morales and Boekel, 1997; Gangadhaiah, *et al*., 2010). Many reports characterizing and monitoring the structural changes of glycated proteins have appeared in the literature (Schmitt, *et al*., 2005; Dutta *et al*., 2006; De Sa, *et al*., 2006). AGEs play an important role in the pathophysiology of diabetes, cataract formation, Alzheimer's disease, renal dysfunction and coronary heart disease [Vitek, *et al*., 1994; Ulrich and Cerami, 2001; Ahmend, *et al*., 1997).

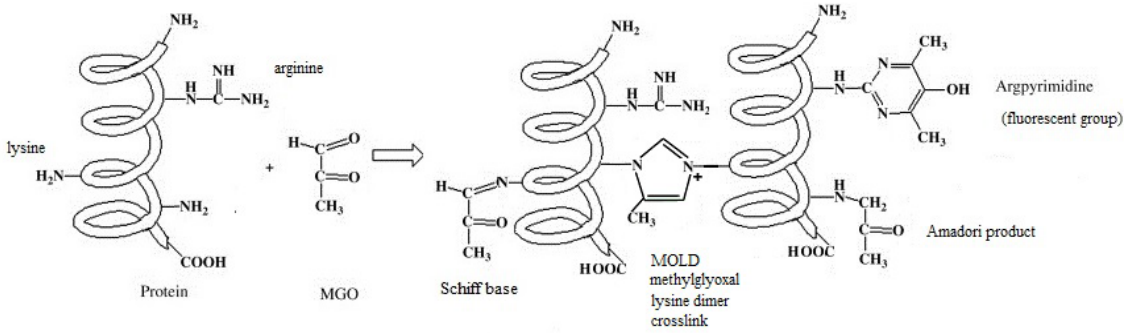


FIGURE 1 MAILLARD REACTION PATHWAY OF METHYLGLYOXAL AND PROTEIN SIDE CHAIN, LYSINE AND ARGININE RESIDUES, LEADING TO THE FORMATION OF SCHIFF BASE PRODUCTS, AMADORI PRODUCTS AS WELL AS MOLD, METHYLGLYOXAL LYSINE DIMER CROSSLINKS AND THE FLUORESCENT ARGININE BASED ARGPRYIMIDINE PRODUCT. DRAWING MODIFIED FROM (Gangadhaiah, *et al.*, 2010).

Methylglyoxal (MG) is a small molecular weight, highly reactive, 3-carbon, reducing glucose metabolite with an aldehyde and a ketone group (CH_3-CO-CHO); and is produced as a breakdown product of several metabolic pathways, mainly from dihydroxyacetone phosphate and D-glyceraldehye 3-phosphate in glycolysis (Price and Knight, 2009). Elevated levels of MG are found in diabetic patients due to excess glucose metabolism (Thornalley, 2008). The formation of AGEs by the reaction of proteins with MG has been well documented (Thornalley, 1996; Fukunaga, *et al.*, 2005; Karachalias, *et al.*, 2003); however, the direct cross-linked, high molecular weight products of protein are rarely detected (Dyer, *et al.*, 1991; Zhang, *et al.*, 2004; Feng, 1997). Previous studies from this laboratory have demonstrated the nonenzymatic formation of AGEs of MG with a wide variety of proteins and amino acids as well as DNA nucleosides and nucleotides (Dutta, *et al.*, 2007; Dutta, *et al.*, 2008; Pampati, *et al.*, 2011).

Human fibrinogen (hFib) is a member of a group of important proteins in the human body involved in the blood clotting cascade. Human fibrinogen (MW 340 kD) is a dimer composed of Aα, Bβ and λ subunits that circulate in the blood stream (Ray, 2000). Each subunit possesses some degree of α-helical content. Human fibrinogen concentration is increased in patients with diabetes, hypertension, hyperlipoproteinaemia, and among smokers (Ang, *et al.*, 2008). Very little information is available concerning the in-vivo interaction of high hFib levels and high sugar contents in diabetic patients. Changes in the integrity of human fibrinogen structure may play a crucial role in the somatic effect of diabetes, hypertension, coronary heart disease or Alzheimer patients. The objective of this project was to investigate the in-vitro formation of the AGEs species of human fibrinogen using supra-physiologically concentrations of MG.

Materials and Methods

Reagents and Supplies

Human fibrinogen fraction I, methylglyoxal 40% (w/w), sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, potassium chloride, sodium azide, and sinapinic acid as well as HPLC grade acetonitrile were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Nanopure grade water was used throughout these analyses. Disposable UV-transparent (12.5x12.5x36 mm) cuvettes were obtained from Hellma (Mullheim, Germany).

Preparation of Buffers and Reaction Mixtures

All in-vitro reactions were conducted in 0.2 M phosphate buffer at pH 7.20 ± 0.05. Sodium azide, 3 mM (0.02%) was added to prevent microbial growth. Four concentrations of MethylGlyoxal stock solutions (40, 20, 10 and 2 mM) and one constant concentration of hFib stock (4 mg/mL) were prepared separately. Each set of four different concentrations of MG was then mixed with equal volume of protein solution to obtain the final MG concentrations of 20, 10, 5 and 1 mM with a constant 2 mg/mL human fibrinogen in the reaction mixtures respectively. Controls included 20 mM MG and 2 mg/mL hFib. The lowest MG concentration is more than 100:1 molar ratio of MG to hFib protein. All mixtures and controls were incubated in the dark at 37^{o}C on a shaker for up to 21 days. Once removed from the incubation, each sample was frozen at -20^{o}C until analysis.

UV & Fluorescence Spectroscopy

The UV spectral scans were performed from wavelength 190 nm to 340 nm with an Ultrospec 2100 pro UV/visible spectrophotometer (GE Healthcare). Fluorescence emission spectroscopy was performed with a Perkin Elmer LS 55 luminescence spectrometer (Shelton, CT, USA) that was equipped with a thermal cell to maintain the samples at 25 ± 1 ^{o}C. All samples were analyzed at an excitation and an emission wavelength of 360 nm and 460 nm respectively. All UV/fluorescence readings were performed in triplicate and the data were reported as the average of these three readings.

High-Performance Liquid Chromatography (HPLC)

HPLC was performed with a gradient system (Hitachi High Technologies America, San Jose, CA, USA) consisting of a high-pressure pump (L-7100), a four-channel degasser, a sequential auto-sampler (L-7200), and a high-sensitivity diode array detector (190-800 nm). AGE species were separated on a COSMOSIL C_{18} packed column of 300 Å pore size and 5 μm particle size with 4.6 mm x 250 cm dimension. Mobile phase 'A' consisted of 100% Nano pure water and that of 'B' was 100% acetonitrile. A gradient condition consisting of 0% to 50% mobile phase 'B' was applied for 15 minutes at a constant flow rate of 1.0 mL/min. Chromatograms were collected at 274 nm, which represents a compromise due to changing λ_{maxes} of the incubated samples. All HPLC runs were repeated three times to ensure reproducibility of the data and peak retention times.

Circular Dichroism Spectrometry (CD)

CD studies were performed on a Jasco J-810 spectro-polarimeter equipped with a Peltier thermoelectric-type temperature controller with an N_2 purging system. The instrument was controlled by Jasco's Spectra Manager™ software. The concentration of hFib was adjusted to 200 μg/ml with 0.2 M phosphate buffer of pH 7.20 ± 0.05. The measurement range was 200–280 nm, and the temperature was kept constant at 20 ± 1 ^{0}C. A quartz cell with a path length of 0.1 cm was used for each run and the analysis was done at a scanning speed of 50nm/min. A total of 10 spectra was obtained and averaged.

Results

Reaction Mixtures

Immediately, upon mixing each of the four concentrations of MethylGlyoxal stock solutions (40, 20, 10 and 2 mM) with the stock hFib solution (4 mg/mL), some degree of a gelatinous precipitate was formed in a concentration dependent manner. 40 mM stock MG formed the most precipitate and 2 mM stock MG formed almost no precipitate. Control solutions of 20 mM MG alone and 2 mg/mL hFib alone did not form a gelatinous precipitate. By day-4 of incubation in the dark at 37 ^{o}C on a shaker, all controls and the test mixtures were cleared as the gelatinous precipitate dissolved. The resulting incubated solutions, day-4 to day-20 were used for the experiments listed below, except in the HPLC experiments where only the clear supernatant was used before day-4 of incubation.

Spectrometry

Human fibrinogen exhibited two characteristic UV λ_{maxes}, one near 231 nm and the other near 280 nm. The λ_{max} near 231 nm is due to the degree of protein folding and the peak near 280 nm is due to Tyr, Phe and Trp content of the protein primary chain. Both of these λ_{maxes} showed a time dependent increase in absorption (hyperchromic shifts) in the hFib-MG reaction mixtures. There was a hyperchromic shift at λ_{max} 231 nm seen only with the highest concentration of 20mM MG, data not shown. This absorption increase provided the preliminary evidence of hFib-MG adduct formation in the reaction mixtures. The change in peak absorbance near 231 nm was time dependent with the greatest change at 21 days incubation at 37^{o}C in the dark. At less than 20 mM MG, the absorption peak at 231 nm was not prominent and did not show detectable absorption changes with time. This suggested that at lower MG concentrations the interaction between MG and human fibrinogen did not result in appreciably amounts of fibrinogen-MG-fibrinogen crosslinking, which was hypothesized to be necessary for AGE formation.

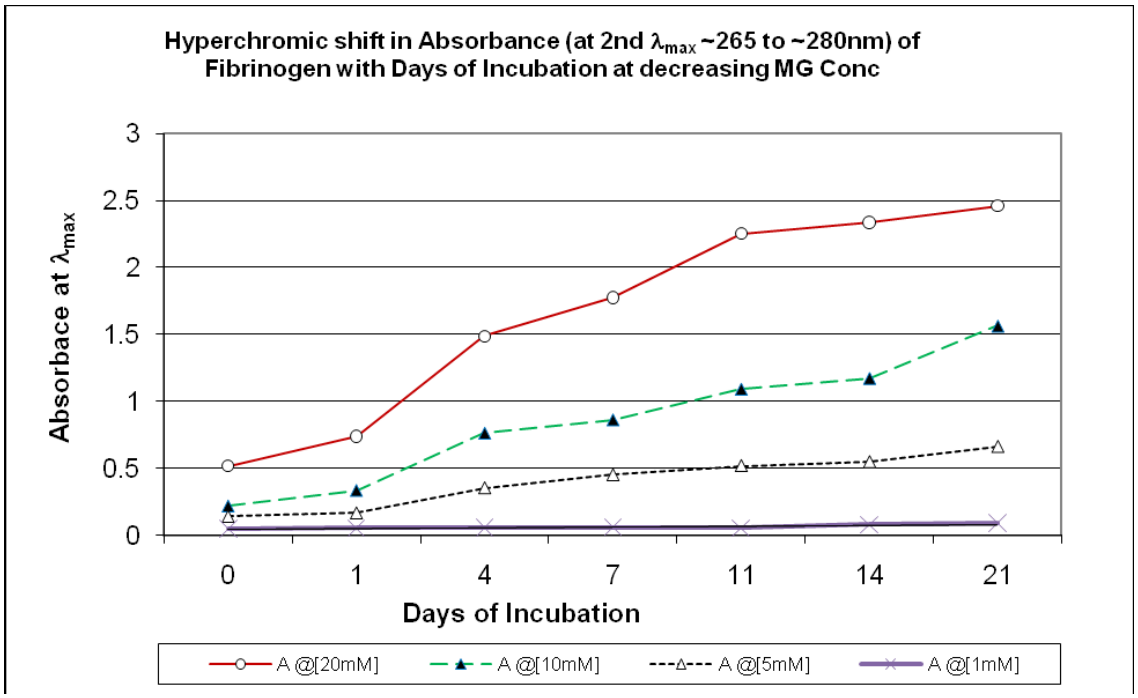


FIGURE 2 UV HYPERCHROMIC SHIFT OF HUMAN FIBRINOGEN (AT THE SECOND λ_{max}) WITH DAYS OF INCUBATION AT VARIOUS METHYLGLYOXAL CONCENTRATION. HIGHER MG CONCENTRATION PRODUCED RELATIVELY HIGHER CHANGE OF ABSORPTION WITH TIME, WHICH FAVORED THE FORMATION OF LIGHT ABSORBING AGES COMPOUNDS. MG CONCENTRATION OF 1 MM OR BELOW VIRTUALLY DID NOT HAVE ANY EFFECT ON THE CHANGE IN ABSORPTION. EACH SAMPLE WAS MEASURED IN TRIPLICATE AND AVERAGED (EACH SET OF OF THE THREE READINGS WERE WITHIN 5% OF EACH OTHER).

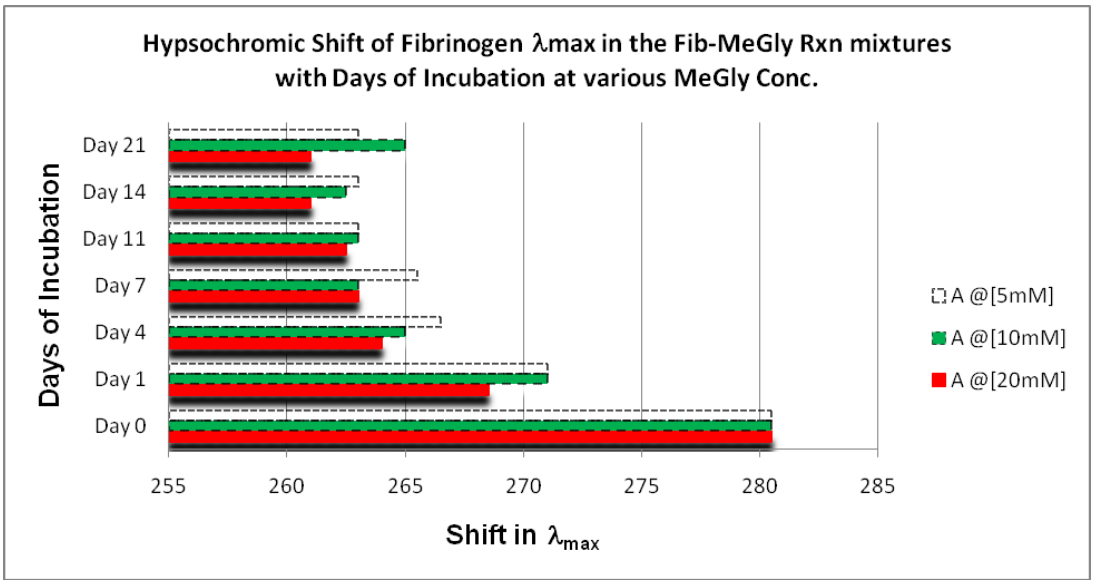


FIGURE 3 UV HYPSOCHROMIC SHIFT OF HUMAN FIBRINOGEN (AT THE SECOND λ_{max}) WITH DAYS OF INCUBATION WITH VARIOUS METHYLGLYOXAL CONCENTRATION.

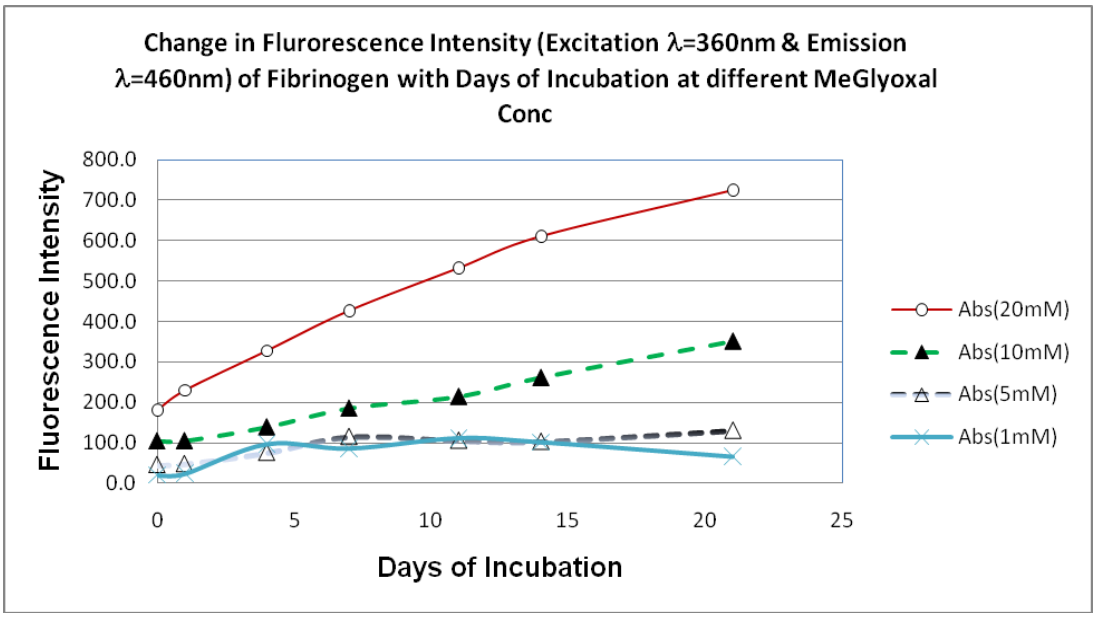


FIGURE 4 INCREASE OF FLUORESCENCE INTENSITY OF FIBRINOGEN WITH DAYS OF INCUBATION AT VARIOUS METHYLGLYOXAL CONCENTRATION, (Excitation λ = 360 nm and Emission λ = 460 nm)

Absorption at the 280 nm showed a concentration dependent increase with MG, Figure 2. The MG concentrations were 20, 10, 5 and 1 mM respectively. Higher MG concentrations and longer incubation time favored the formation of light absorbing AGE compounds. Decreased MG concentrations reduced the formation of AGEs with essentially no reaction when the MG concentration was as low as 1mM as demonstrated in Figure 2.

Figure 3 showed the hypsochromic (or blue) shifts of the absorption λ_{max} of the hFib-MG reaction mixtures as a function of time at various MG concentrations. The λ_{max} of the incubated reactants shifted from near 280 to near 260 nm during the 21 days of incubation. This blue shift was believed to demonstrate the formation of newer and/or stronger bonded compounds. Figure 3 also showed that higher MG concentration produced larger hypsochromic shifts compared to lower MG concentrations. This suggested that higher MG concentration plays a greater role in the increased formation of AGE species. Thus the hypsochromic shifts along with the rising absorptions of the reaction mixtures reinforced a the evidence of in-vitro AGEs formation.

The fluorescence profile, Figure 4, exhibited a similar trend as UV except the change in fluorescence intensity was much higher compared to the change in

UV. The lowest MG concentration essentially showed no change in fluorescence pattern of the reaction mixture. This was consistent with the UV data which reinforced the fact that AGE species formation stops when the MG concentration approaches toward 1 mM as demonstrated in Figure 2.

In summary, the UV data showed the increase in absorption of the reaction mixtures. This change in the system was further enhanced by a hypsochromic shift to shorter wavelengths, which indicated the formation of either new bonds or stronger bonded compounds with the progression of time. Fluorescence data further verified the UV result for the formation of AGEs. Both UV and fluorescence results suggested that the distinct light absorbing AGEs of human fibrinogen are directly proportional to the MG concentration and that a threshold amount of MG is required for such formations of AGE species.

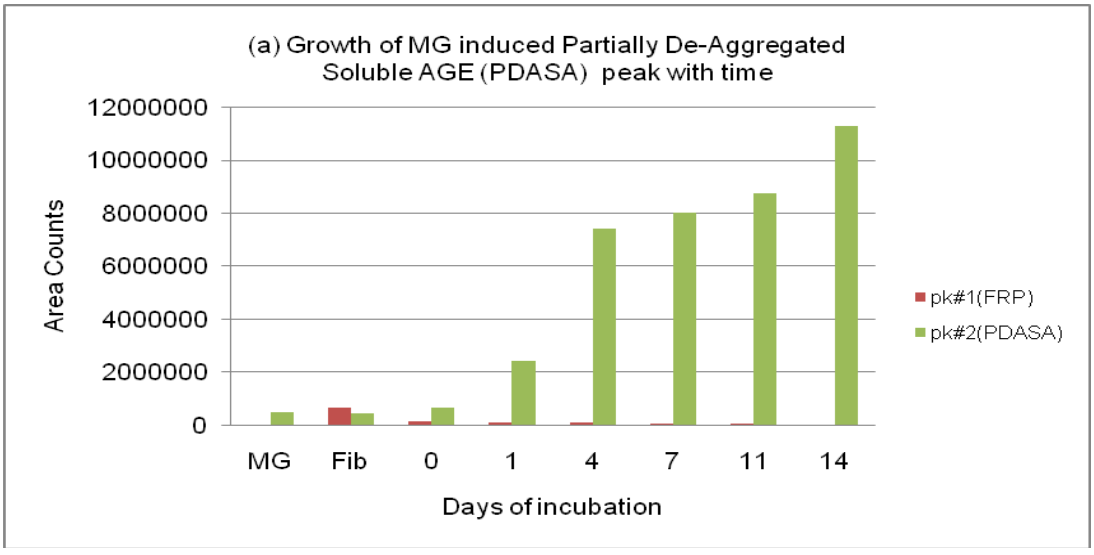
HPLC Analysis

The area counts of different peaks assigned as hFib and MG controls as well as for the he reaction products are tabulated in Table 1. The chromatogram of hFib control produces two peaks (pk#1 and pk#2), while that of MG controls was associated with three peaks (pk#3, #4 and #5). Table 1 also shows the increase in area of peak#2 [hFib/MG (PDASA) peak]. There were concurrent decreases in peak#1 (hFib only) and the MG only peaks (pk#3, 4 and 5). This suggests that addition of MG formed an adduct with fibrinogen. These new compounds at peak #2, increased in quantity (indicated by the increase in area counts), while the MG only peaks decreased in area counts.

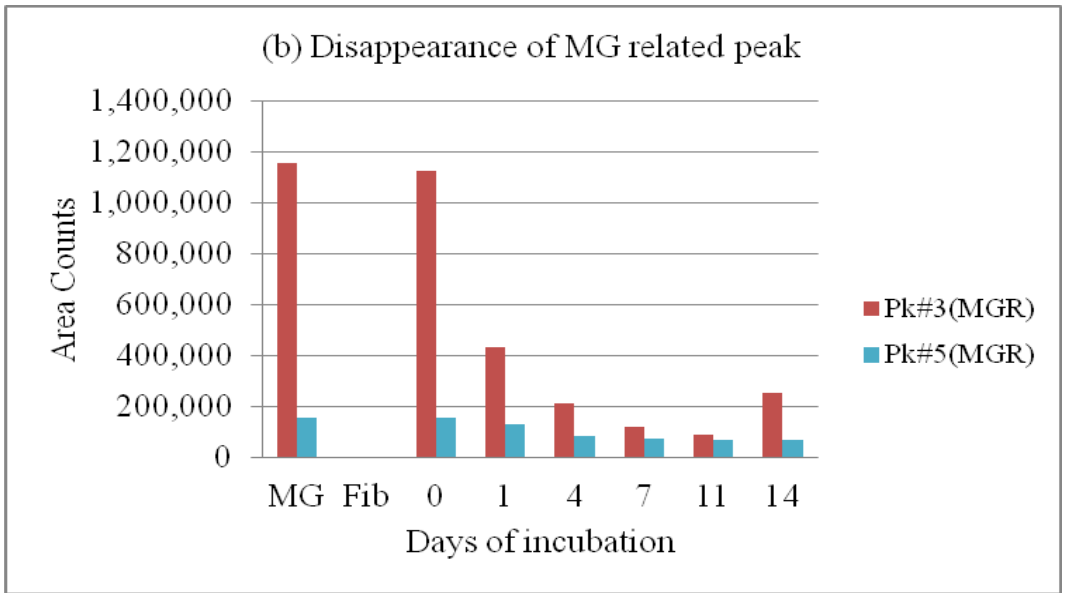
TABLE 1 THE PEAK AREA COUNTS OF HUMAN FIBRINOGEN AND MG CONTROLS. THE PEAK AREA COUNTS OF hFib-MG REACTION MIXTURES WITH THE PROGRESSION OF INCUBATION TIME. SAMPLES WERE INJECTED IN TRIPLICATE AND THE TOTAL AREAS WERE AVERAGED. THE BLANK PHOSPHATE BUFFER RUN PRODUCED A CO-ELUTING PEAK AT THE SAME RETENTION TIME WITH pk#2, SO THE AREA FOR pk#2 FOR ALL THE SAMPLES WAS CALCULATED AFTER SUBTRACTING FROM THE AREA COUNTS OF THE RESPECTIVE BLANK PEAK.

	hFib	hFib and (**PDASA**)	MG	MG	MG
RT=> Samples	2.8 min(pk#1)	3.6 min(pk#2)	4.0 min(pk#3)	4.5 min(pk#4)	5.8min(pk#5)
Fib Control	645,396	453,040	---------	---------	--------
MG Control	---------	---------	1,157,623	26,576	156,369
Fib-MG- 0 day	141,159	646,686	1,126,853	21,707	156,487
Fib-MG- 1 day	91,310	2,405,646	430,444	6,544	129,270
Fib-MG- 4 day	90,026	7, 405,646	211,076	3,796	84,806
Fib-MG- 7 day	59,265	8,005,646	117,661	4,528	75,984
Fib-MG- 11 day	51,205	8,768,213	90,808	4,641	70,484
Fib-MG- 14 day	D	11,300,935	251,013	120,692	67,405

D= Peak disappeared; PDASA=Partially De-Aggregated Soluble AGE peak; MG= Methyl Glyoxal Related Peak;



(a) the growth of MG induced Partially De-Aggregated Soluble AGE (PDASA) peak. Peak#1 of hFib (shown in red) started to disappear after mixing with MG and the pk#2 (shown in green), the PDASA peak sharply increased after day-4 when the aggregated Insolube pre-AGE (AIsp-A) started to de-aggregate in to the solution with time.



(b) Showed the disappearance of MG peak. MG depleted with days of incubation suggesting that MG is consumed in the AGE formation with Fib or with its subunits.

FIGURE 5 HPLC DATA SHOWING

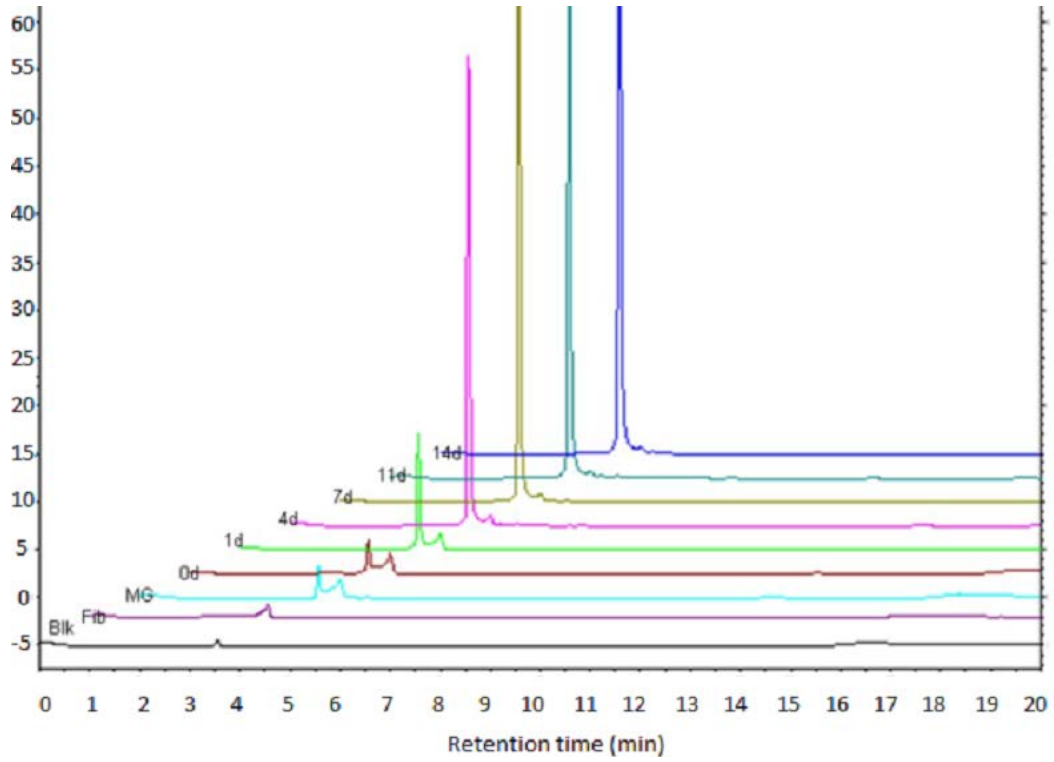


FIGURE 6 OVERLAY HPLC PROFILE OF ALL THE REACTION MIXTURES INCLUDING BLANK PHOSPHATE BUFFER, BLANK FIBRINOGEN, METHYLGLYOXAL BLANK AND ALL THE REACTION PRODUCTS OF INCUBATED SAMPLES SHOWING THE RELATIVE INCREASE OF PDASA PEAK AS THE REACTION PROGRESSES. THE PDASA PEAK INCREASES SHARPLY AFTER DAY-4.

Figure 5(a) showes the increased formation of the PDASA species. Figure 5(b) on the other hand shows the decrease in MG peak, suggesting that the free MG is bound in the formation of hFib-MG AGEs by interaction with the protein. The HPLC data supports the formation of PDASA compounds which come from the breakdown of the initial gelatinous, aggregated, insoluble glycation products. Figure 6 displays the overlay chromatograms of all the reactants along with the hFig-MG product with time. The sharp increase of the PDASA peak area (pk#2) is clearly visible from day-4 onwards as the gelatinous precipitate fully de-aggregates in solution.

Circular Dichroism (CD)

Figure 7 presents the CD spectrophotometric profile of

the reaction mixtures from the initial time point through 21 days. Native fibrinogen possesses a pronounced α-helix structure as shown by the characteristic double negative dip at 220 and 213 nm. The CD spectrum taken from the supernatant at day-0 showed no α-helical fibrinogen in it. This was presented to demonstrate that all α-helical fibrinogen in the reaction mixture has fully participated in the formation the aggregated insoluble gel immediately after coming in contact with MG. The lack of a dip in the day-0 CD spectrum further suggested that hFib in the day-0 HPLC analysis is non-α-helical in nature. The gel began to de-aggregate from day-4 onwards into PDASA species. The PDASA formed at day-4 possessed partial α-helical structure as indicated by the re-appearance of the double dip in the CD spectrum. Further CD analysis of later incubation aliquots illustrated that the α-helical form of the PDASA continued to increase with time, however, it did not completely return to the original native fibrinogen α-helical form even up to 3 weeks of incubation.

Discussion

Human fibrinogen is a large (340kD) soluble glycoprotein containing three pairs of polypeptide chains: Aα (66kD, 610 amino acids), Bβ (54kD, 461 amino acids) and γ (48kD, 411 amino acids) and synthesized in the liver as a dimer $(A\alpha\text{-}B\beta\text{-}\gamma)_2$ and circulates in the blood plasma at a concentration of 2-4 mg/ml (Ray, 2000). Human fibrinogen levels are higher ($\geq$3.75 mg/ml) in patients with diabetes mellitus (Ang, *et al.*, 2008) and hypertension ($\geq$3.9 mg/ml) (Shankar, *et al.*, 2006). An elevated level of fibrinogen in the blood serum is a risk factor for coronary heart disease including stroke, ischemia and other age related diseases (Onohara, *et al.*, 2000)]. Human fibrinogen as a precursor of fibrin may be involved in the diabetic complications in hyperglycemic patients (Ceriello, 1997).

While in-vivo glycation of hFib with glucose is known to form AGE products (Zhang, *et al.*, 2004), very little attention has been given to the glycation of hFib by the glucose metabolite, MethylGlyoxal, which also forms AGEs. MG is formed in-situ from a number of metabolic pathways in the body, mainly glyolysis (Price and Knight, 2009) and can be predicted to form AGEs since it contains the same functional aldehyde and ketone groups as in glucose and other reducing sugars. Glycation of hFib by MG may cause severe effects on diabetes and coronary heart disease. In this current report, we presented evidence of hFib AGEs formation with MG supported by multiple techniques. Although, many reports of AGE formation with various proteins can be found in the literature, this is the first report that the addition of MG induces Fibrinogen to form in-vitro AGE species.

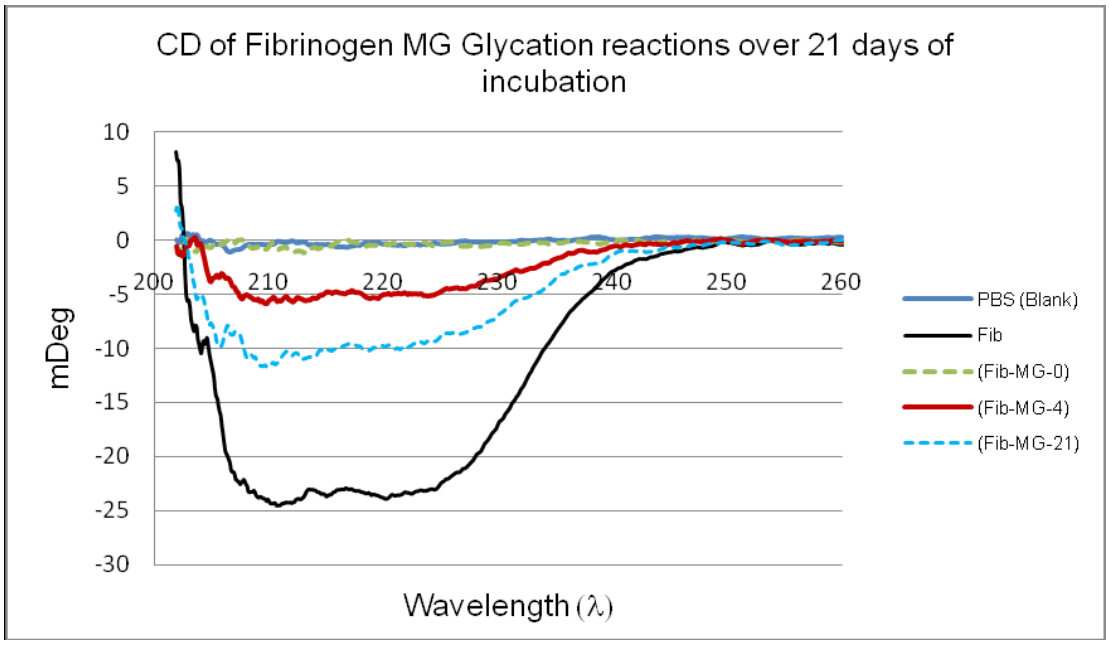


FIGURE 7 CIRCULAR DICHROISM (CD) SPECTRA OF HFIB AND HFIB-MG INCUBATED REACTION MIXTURES AT DAY 0, 4 AND 21. A TOTAL OF TEN SPECTRA WERE COLLECTED AND AVERAGED. THE DOUBLE HUMP NEGATIVE DIP AT 220 AND 213 NM INDICATED THAT THE NATIVE PROTEIN HAS A α-HELIX STRUCTURE. THE SUPERNATANT OF THE ZERO DAY SAMPLE SHOWED NO A-HELICAL PROTEIN. AT DAY-4, THE PROTEIN IN SOLUTION HAS SIGNIFICANTLY LOST ITS A-HELICAL FORM. THE α-HELICAL STRUCTURE OF THE PROTEIN IS REGAINED AT DAY-21 OF INCUBATION; HOWEVER COMPLETE RESTORATION OF THE ORIGINAL SECONDARY STRUCTURE WAS NOT OBTAINED EVEN AFTER 21 DAYS OF INCUBATION.

The data presented here shows the in-vitro formation of AGE species using normal physiological levels of hFib and supranormal concentrations of MG. These higher MG concentrations were utilized in order to see the upper boundary effects. It is not known if these high levels of MG are possible even in the extreme cases of uncontrolled diabetes. It would certainly be disastrous if the changes that we report with in-vitro gelatinous precipitate formation occur in-vivo.

On day-0 the initial mixing of hFib-MG formed a gelatinous precipitate, which is an aggregated product of hFib and MG. It is known that the primary binding site of MG to a protein is probably at the arginine residues. This allows for both oxygen atoms of the MG molecule to attach to two of the three nitrogen atoms on the same guanidinium group (Park, *et al.*, 2003; Ahmed, *et al.*, 2005). This adduct probably does not go on to form further cross-links, but it may form argpyrimidine [Nδ-(5-hyroxy-4,6dimethylpyrimidin-2-yl)-L-ornithine] a fluorescent product (Shipanova, *et al.*, 1997).

Because these experiments used such a large excess of MG, the lysine residues were probably also glycated. Modification at lysine residues does allow for an Amadori rearrangement and intramolecular crosslinks as shown in Figure 1. Formation of intermolecular crosslinks at the lysine residues probably is the cause of the gelatinous precipitate, which remained visible for a few days and then disappeared by day-4 of incubation. It was suggest that MG induced a conformational change in the human fibrinogen which caused auto-aggregation and precipitation. At this time, it is postulated that the MG, a bi-dentate Schiff base ligand, was able to bridge across two hFib molecules and thereby crosslinked them to form a high molecular weight aggregate of insoluble pre-AGEs on day-0. Since this aggregated material dissolved by day-4, it was assumed that the MG formed unstable Schiff bases attachments with surface lysine residues. Some of these unstable Schiff base formations were rehydrated by day-4 which allowed the hFib molecules to be released and partially renature as seen by CD. It is not known if this type of precipitate is formed in-vivo, but it probably does not as very high MG levels were investigated here. Since MG is such a reactive molecule, and human fibrinogen has a 4 day half-life in the body (Haidaris, *et al.*, 1989) this high level of glycation in vivo has not been reported.

Based on our in vitro experiments, it is therefore proposed that MG induces hFib to form at least two different but related kinds of glycation products. (i) The gel-like, Aggregated Insoluble pre-AGE (AIsp-A) which is stable up to 4 days. (ii) The Partially De-Aggregated Soluble AGEs (PDASA), which appears to increase sharply on day-4 as shown by the increase of the existing Fib HPLC pk#2, which continues to increase at least up to 14 days of incubation. There may even be evidence of a third type of AGE that is a Non-Aggregated Soluble AGEs (NASA) that may be formed between MG and some small hFib subunit, possibly a single Aα , Bβ or γ subunit. This third AGE type remains soluble and is first seen on day-1 (not shown here) and remains an area of ongoing research in this laboratory.

The Aggregated Insoluble pre-AGE, AIsp-A form is the insoluble gelatinous precipitate which may actually be a new model of a re-soluble fibrin type clot. It is resoluble because there are no other proteins present to lockdown the insoluble clot conformation. Since the equilibrium of Schiff base formation is highly reversible in an aqueous environment, it is possible that one of the two attachment sites on MG rehydrates and releases the hFib lysine site. Once released, this allows the hFib-MG complex to assume a more native hFib conformation. At this time, the molecular weight of the soluble PDASA hFib-MG complex is not known. Our observations have indicated that once the PDASA material is resolubilized from the initial gelatenous precipitate, it remains soluble even while non-specifically binding more MG molecules.

In summary, UV, Fluorescence, HPLC, and CD support the formation of aggregated and non-aggregated AGEs of Fibrinogen with MG. Direct visual observation of the gelatinous precipitate at initial mixing indicates the presence of the Aggregated Insolule pre-AGE, hFib-MG species. HPLC analysis revealed that MG induces the hFib to form the PDASA types of AGEs. The AIsp-A remains insoluble for up to 4 days. From day 4 onwards, AIsp-A begins to de-aggregate partially into soluble PDASA species which continues to increase in amount until at least 14 days. The mechanism by which the AIsp-A turns to PDASA remains to be investigated. CD profiles demonstrated that the MG induced fibrinogen AGE is denatured relative to native Fib. Although part of the denatured PDASA reversibly re-natured to α-helical form from day-4 to day-21, the current data also showed that PDASA aggregates do not fully renature even after 3 weeks.

We have demonstrated the formation of both pre-

advanced glycation end products of hFib with MG and AGEs supported by spectroscopy and other powerful analytical tools. The non-enzymatic in vitro reaction of hFib with MG warrants further investigation by other sugars which can also be toxic glycators and play a crucial role in the polymerization of AGE products that can lead to pathological complications in diabetes and coronary heart disease. Also the Alsp-A hFib-MG, itself, warrants further investigation as a re-soluble clot model for future in vitro study.

ACKNOWLEDGEMENTS

This research was jointly supported by NCRR/NIH Grant # P20 RR016457 and the Network institutions. The authors thanked RI-INBRE core facility and Dr. Aftab Ahmed (URI-INBRE Manager) for providing the instrumentation required to carry out this research work.

REFERENCES

Ahmed, M.U., E. Brinkmann Frye, T.P. Degenhardt, S.R. Thorpe, J.W. Baynes, N^{ε}-(Carboxyethyl)lysine, a product of chemical modification of proteins by methylglyoxal, increases with age in human lens proteins, Biochem. J., 324, (1997) 565–570.

Ahmed, N., D. Dobler, M. Dean, P. J. Thornalley, Peptide Mapping Identifies Hotspot Site of Modification in Human Serum Albumin by Methylglyoxal Involved in Ligand Binding and Esterase Activity. J. Biol. Chem., 280 (7), (2005) 5724-5732.

Ang, L., V. Palakodeti, A. Khalid, S. Tsimikas, Z. Idrees, P. Tran, P. Clopton, N. Zafar, G. Bromberg-Marin, S. Keramati, E. Mahmud, Elevated Plasma Fibrinogen and Diabetes Mellitus Are Associated With Lower Inhibition of Platelet Reactivity With Clopidogrel. J Am Coll Cardiol, 52, (2008) 1052-1059.

Ceriello, A. Fibrinogen and diabetes mellitus: is it time for intervention trials? Diabetologia 40, (1997) 731–734.

De Sa, P.F.G., J.M. Treubig, P.R. Brown, J.A. Dain, The use of capillary electrophoresis to monitor Maillard reaction products (MRP) by glyceraldehyde and the epsilon amino group of lysine, Food Chem. 72, (2001) 379-384.

Dutta, U., M.A. Cohenford, J.A. Dain, Monitoring the effect of glyceraldehyde glycation on the secondary structure human serum albumin and immunoglobulin G: an analysis based on circular dichroism, thermal melting profiles, and UV–fluorescence spectroscopy, Anal. Chim. Acta., 558, (2006) 187-194.

Dyer, D.G., J. A. Blackledge, S. R. Thorpet, J. W. Baynes, Formation of Pentosidine during Nonenzymatic Browning of Proteins by Glucose, J. Biol. Chem. 266, (18) (1991) 11654-11660.

Feng, L. Nonenzymatic Glycation of Human Fibrinogen and Myelin Basic Protein, PhD dissertation, University of Rhode Island, 1997.

Fukunaga, M., S. Miyata, S. Higo, Y. Hamada, S. Ueyama, M. Kasuga, Methylglyoxal induces apoptosis through oxidative stress mediated activation of p38 mitogen-activated protein kinase in rat Schwann cells, Ann. N. Y. Acad. Sci. 1043, (2005) 151–157.

Gangadhaiah, M.H., B. Wang, M. Linetsky, C. Hemming, R. Spanneberg, Hydroimidazolone modification of human αA-crystallin: Effect on the chaperone function, and protein refolding ability, Biochim et Biophys Acta, 1802, (2010) 432-441.

Haidaris, P. J., C.W. Francis, L.A. Sporn, D.S. Arvan, F.A. Collichio, V.J. Marder, Megakaryocyte and Hepatocyte Origins of Human Fibrinogen Biosynthesis Exhibit Hepatocyte-Specific Expression of Gamma Chain-Variant Polypeptides. Blood, 74, (1989) 743–50.

Karachalias, M., R. Babaei-Jadidi, N. Ahmed, P.J. Thornalley, Accumulation of fructosyl-lysine and advanced glycation end products in the kidney, retina, and peripheral nerve of streptozotocin-induced diabetic rats, Biochem. Soc.Trans. 31, (2003) 1423–1425.

Li, Y., U. Dutta, M.A. Cohenford, J.A. Dain, Nonenzymatic Glycation of Guanosine 5′ Triphosphate by Glyceraldehyde: An in Vitro Study of AGE Formation, Bioorg. Chem. 35, (6) (2007) 417-429.

Li, Y., U. Dutta, M.A. Cohenford, J.A. Dain, The structural modification of DNA nucleosides by nonenzymatic glycation: an in vitro study based on the reactions of glyoxal and methylglyoxal with 20-deoxyguanosine, Anal. Bioanal.Chem. 390, (2008) 679–688.

Maillard, L.C., Action des acides amines sur les sucres: Formation des melanoidines par voie methodologique, Compt. Rend. Hebd. Acad. Sci. 154 (1912) 66-68.

Morales, F.J., M.A.J.S. van Boekel, A Study on Advanced Maillard Reaction in Heated Casein/Sugar Solutions:

Fluorescence Accumulation, Int. Dairy J. 7, (1997) 675-683.

Onohara. T., K, Komori, M. Kume, M. Ishida, S. Ohta, K. Takeuchi, T. Matsumoto, K. Sugimachi. Increased plasma fibrinogen level and future risk of coronary artery disease after repair of abdominal aortic aneurysm. J. Am. Coll. Surg., 191 (6), (2000) 619-25.

Pampati, P. K., S. Suravajjala, J. A. Dain, Monitoring nonenzymatic glycation of human immunoglobulin G by methylglyoxal and glyoxal: A spectroscopic study, 408, (2011) 59–63.

Park, Y. S., Y.H. Koh, M. Takahashi, Y. Miyamoto, K. Suzuki, N. Dohmae, K. Takio, K. Honke, N. Taniguchi, Identification of the binding site of methylglyoxal on glutathione peroxidase: methylglyoxal inhibits glutathione peroxidase activity via binding to glutathione binding sites Arg 184 and 185. Free Rad. Res. 37 (2), (2003) 205-211.

Price, C.L., S. C. Knight, Methylglyoxal: Possible Link Between Hyperglycaemia and Immune Suppression, Trends in Endocrinology and Metabolism 20, (2009) 312-317.

Ray, A., A SAF Binding Site in the Promoter Region of Human alpha-Fibrinogen Gene Functions as an IL-6 Response Element 1, J. Immunol. 165, (2000) 3411-3417.

Schmitt, A., J. Schmitt, G.Munch, J. Gasic-Milencovic, Characterization of advanced glycation end products for biochemical studies: side chain modifications and fluorescence characteristics, Anal. Biochem. 338, (2005) 201-215.

Shankar, A., J.J. Wang, E. Rochtchina, P. Mitchell, Positive Association Between Plasma Fibrinogen Level and Incident Hypertension Among Men, *Hypertension,* 48, (2006) 1043.

Shipanova, I.N., M.A. Glomb, R.H. Nagaraj. Protein modification by methylglyoxal: chemical nature and synthetic mechanism of a major fluorescent adduct. Arch. Biochem. Biophys. 1997;344:29–36.

Thornalley, P.J. Pharmacology of methylglyoxalase: formation, modification of proteins and nucleic acids, and enzymatic detoxification: a role in pathogenesis and antiproliferative chemotherapy, Gen. Pharmacol. 27, (1996) 565–573.

Thornalley, P.J., Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems: role in ageing and disease, Drug Metab. Drug Interact. 23, (2008) 125–150.

Ulrich, P., A. Cerami, Protein glycation, diabetes, and aging. Recent Prog Hormone Res. 56, (2001) 1–21.

Vitek, M.P., K. Bhattyacharya, J. M. Glendening, E. Stopa, H. Vlassara, R. Bucala, K. Manogue, A. Cerami, Advanced glycation end products contribute to amyloidosis in Alzheimer disease. Proc. Natl. Acad. Sci. 91, (11). (1994) 4766–4770.

Wolff, S. P., Z.Y. J.V. Jiang, Hunt, Protein glycation and oxidative stress in diabetes mellitus and ageing., Free Rad. Biol. and Med., 10 (5), (1991) 339-352.

Zhang, X., Y. Ma, H. Liu, P.F.G.De Sa, P.R. Brown, J.A. Dain, Monitoring the formation of Maillard reaction products of glucosamine with fibrinogen and human serum albumin using capillary electrophoresis, Anal. Biochem., 325, (2004) 255-259.